Prolonged Hypoxia Concomitant with Serum Deprivation Induces Massive Human Mesenchymal Stem Cell Death

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Abstract: Mesenchymal stem cells (MSCs) have been proposed for the repair of damaged tissue including bone, cartilage, and heart tissue. Upon in vivo transplantation, the MSCs encounter an ischemic microenvironment characterized by reduced oxygen (O_2) tension and nutrient deprivation that may jeopardize viability of the tissue construct. The aim of this study was to assess the effects of serum deprivation and hypoxia on the MSC survival rates *in vitro*. As expanded MSCs are transferred from plastic to a scaffold in most tissue engineering approaches, possibly inducing loss of survival signals from matrix attachments, the effects of a scaffold shift on the MSC survival rates were also assessed. Human MSCs were exposed for 48 hours to (i) a scaffold substrate shift, (ii) serum deprivation, and (iii) O_2 deprivation. MSCs were also exposed to prolonged (up to 120 hours) hypoxia associated with serum deprivation. Cell death was assessed by Live/Dead staining and image analysis. The MSC death rates were not affected by the shift to scaffold or 48-hour hypoxia, but increased with fetal bovine serum (FBS) starvation, suggesting that between the two components of ischemia, nutrient deprivation is the stronger factor. Long-term hypoxia combined with serum deprivation resulted in the complete death of MSCs (99 ± 1%), but this rate was reduced by half when MSCs were exposed to hypoxia in the presence of 10% FBS (51 ± 31%). These results show that MSCs are sensitive to the concurrent serum and O_2 deprivation to which they are exposed when transplanted *in vivo*, and call for the development of new transplantation methods.

Introduction

The possibility of isolating and expanding autologous mesenchymal stem cells (MSCs) and driving them towards numerous phenotypes including chondrogenic (1-4), osteogenic (2-4), adipogenic (2-4), neural (5, 6) and cardiomyocyte (7) phenotypes, has opened new avenues for repairing lost or damaged tissues. The possible clinical applications include MSCs either in the form of cell suspensions to restore cardiac (7-9) or cerebral (10, 11) function after tissue ischemia, or in association with a biocompatible scaffold for repairing cartilage (12-14), bone (15-17), and adipose (18, 19) tissues.

Although the potential impact of MSC-based therapies is enormous, a number of methodological improvements are still required to be able to repair tissue in clinically relevant volumes and introduce these therapies at clinical level. In fact, an increasing amount of evidence has suggested that the ultimate efficacy of MSC therapy will depend on the possibility of delivering a large number of viable functional cells into injured tissue. Transplantation of MSCs into ischemic heart causes a massive (99%) and rapid (< 4 days) cell death (7), for instance. The reasons for this high peri-transplantation cell death rate are still a matter of conjecture, but experiments in which rat neonatal cardiomyocytes grafted into a vascularized tissue survived better than cells transplanted into ischemic tissues (20) have suggested that some components of ischemia might affect the grafted cell survival rates. We, therefore, hypothesized that two components of ischemia (low oxygen tensions and poor nutrient supply) might be responsible for the extensive cell death observed upon transplantation of MSCs. In addition, engineering a bioartificial tissue conventionally involves detaching MSCs from a cell culture dish and seeding them into a scaffold. It seemed likely that the loss of survival signals from matrix attachments occurring during the cell detachment phase might also impair hMSC survival.

The aim of this study was therefore to assess the *in vitro* effects of (i) low oxygen tensions, (ii) nutrient deprivation and (iii) transferring hMSCs from plastic to scaffold substrates on human MSC (hMSC) survival. Scaffold substrates used were alumina and calcium carbonate, which have previously been used successfully as osteoconductive scaffolds for bone tissue engineering in rabbit (21) and sheep (15) models.

The effects of these parameters were investigated both separately and combined. For this purpose, hMSCs expanded *in vitro* on plastic at 21% O_2 in αMEM/10% fetal bovine serum

(FBS) were exposed to low oxygen tensions, serum deprivation and a change of scaffold. Cell death rates were assessed at various times ranging from 48 to 120 hours.

Materials and Methods

Cell culture

The hMSCs were isolated from the femoral neck in a total of 5 donors (3 males and 2 females, 51-68 years of age) undergoing hip surgery for non-metabolic bone diseases. Patients with osteoarthritis are often used in studies as a control population (22-24) as bone marrow samples from such patients exhibited a maintenance of colony-forming unit fibroblasts (CFU-F) number and CFU-F osteogenic activity as assessed by alkaline phosphatase activity (25) and are of easy access. In our study, they exhibited a normal morphology and potential for proliferation. Although unlikely, a difference in behavior between MSCs issued from normal individual and the ones issued from this subset of patients cannot be excluded. hMSCs were isolated using a procedure previously described in the literature based on their adherence to plastic (26). Briefly, cells were harvested by gently flushing bone marrow samples with alpha Minimum Essential Medium (αMEM, Sigma, Saint Quentin Fallavier, France) containing 10% fetal bovine serum (FBS, PAA Laboratories, Les Mureaux, France) and 1% antibiotic and anti-mycotic solution (PAA Laboratories) and seeded into cell culture flasks. When the hMSCs reached 60-70% confluence, they were detached and sub-cultured (density: 1,000 cells/cm^2). hMSCs were cryopreserved at P1 (90% FBS, 10% dimethyl sulfoxide (DMSO)). A fresh batch of hMSCs was thawed and cultured for each experiment (each passage was run at 5,000 cells/cm^2). Using this expansion protocol, only adherent cell layer was selected. Non adherent hematopoietic cell number decreases with consecutive passages. Cells from each donor were cultured separately. In a previous study, hMSCs isolated from young patient and using the present protocol were able to differentiate along the adipogenic (Oil Red O positive cells, fatty acid binding protein 4 and peroxisome proliferator activated receptor expression), osteogenic (increased alkaline phosphatase activity, osterix and osteocalcin expression) and chondrogenic (type II collagen expression) lineages (Potier et al., revised manuscript, *Bone*).

Scaffold substrates.

Plastic substrate used for cell culture consisted of specially treated polystyrene (Nunclon™, Nunc). Disks (∅= 14 mm) of dense calcium carbonate in a form of aragonite were sawn out of blocks of Tridacna shell and mirror polished. Mirror polished alumina ceramic disks (∅= 14 mm) were provided by Ceraver (Roissy CDG, France)

Serum concentrations

The different serum conditions used were: (i) αMEM + 10% FBS; (ii) αMEM + 1% FBS; and (iii) αMEM + 0% FBS, supplemented with amino acids 1% (Gibco Live Technologies).

Hypoxia

Hypoxia was obtained using a sealed jar (Oxoid Ltd, Basingstoke, United Kingdom) containing an oxygen chelator (AnaeroGen, Oxoid Ltd) (27). Twice a day, the pO_2 was measured directly in the cell culture medium (pH=7.2) using an Oxylab pO_2™ (Oxford Optronix; Oxford, United Kingdom) and without opening the sealed jar. Severe hypoxic conditions (pO_2 < 1%) could be taken to be reached after 48 hours (data not shown). The method used in the present study measured the bulk pO2 in cell culture medium. The pericellular oxygen tension (depending on cell metabolic rates, cell number, diffusion distance of oxygen, and medium stirring) was not evaluated.

Cell death assays

hMSCs (passage P3-P5) expanded onto cell culture plastic substrate were detached using trypsin-EDTA (Sigma) and seeded onto either cell culture plastic (control conditions), alumina, or calcium carbonate substrates in 24-well plates (Nunclon™, Nunc) at a density of 5,000 cells/cm^2. After being left to adhere overnight, cells were washed in PBS and exposed to the experimental conditions for different periods of time. Cells were then stained with the Live/Dead viability/cytotoxicity kit (Molecular Probes, Invitrogen, Cergy Pontoise, France; [calcein AM] = 1 µM in PBS, [ethidium homodimer 1] = 4 µM in PBS), in which nonfluorescent cell-permeant calcein AM is converted into green fluorescent calcein in living cells (due to the presence of intracellular esterase activity), and ethidium homodimer 1 enters and binds to nucleic acids in damaged cells (red fluorescence). Cell death was then assessed using image analysis methods (Leica Qwin software) to count green and red cells. At each count a minimum of 100 cells (either dead or alive) was counted. Each experimental condition was performed in triplicate with each of the donors.

Statistical analysis

Data are expressed as means ± standard deviations. Statistical analysis was performed using an analysis of variance (ANOVA) with a Bonferroni-Dunn post hoc test.

Results

Effects of scaffold substrate shift under standard cell culture conditions

The effects of transferring hMSCs from plastic to either plastic, alumina (21, 28), or calcium carbonate (15) substrates were first investigated separately. In this experiment, oxygen tensions and serum concentrations remained unchanged (i.e. 21% O_2 and 10% FBS). Cell death rates were not affected by the shift from plastic to either plastic, alumina or calcium carbonate substrates.

Scaffold shift per se therefore did not significantly affect the hMSC death rates under standard cell culture conditions.

Effects of serum deprivation

Effects of FBS deprivation per se. To investigate the effects of serum deprivation per se, hMSCs transferred from plastic to plastic were exposed to αMEM containing either 0%, 1% or 10% FBS. Oxygen tensions remained unchanged throughout the experiment (i.e. 21% O_2). Cell death rates increased significantly when hMSCs were exposed to medium containing 0% FBS (13 ± 4%), in comparison with hMSCs exposed to 10% FBS (4 ± 2%; p=0.0042) (Fig. 1A).

Combined effects of FBS deprivation and scaffold substrate. To determine the possible cumulative effects of serum deprivation and the change of scaffold substrate, the above experiment was repeated, but in this case, hMSCs were transferred from plastic to alumina (Fig. 1B) or calcium carbonate (Fig. 1C) substrates. Similarly to what was observed when hMSCs were transferred from plastic to plastic, shifting hMSCs to an alumina substrate significantly increased the cell death rates when the shift was carried out in αMEM containing 0% FBS (12 ± 4%), in comparison with αMEM containing 10% FBS (5 ± 2%; p=0.0082)

(Fig. 1B). Conversely, shifting hMSCs from plastic to a calcium carbonate substrate did not affect the cell death rates at any of the FBS concentrations tested (Fig. 1C).

Combined effects of FBS deprivation under short hypoxia. To simulate more closely the in vivo conditions under which MSCs are transplanted (into an ischemic rather than a well vascularized environment), the combined effects of serum and oxygen deprivation on hMSC survival were investigated. For this purpose, hMSCs (expanded on plastic at 21% O_2 in αMEM/10% FBS) were transferred onto plastic and exposed to 48-hour hypoxia in αMEM containing either 0%, 1% or 10% FBS (Fig. 2A). Results obtained under these conditions were compared to those obtained when hMSCs expanded under the same conditions were transferred from plastic to plastic substrate and exposed to αMEM containing either 0%, 1% or 10% FBS at 21% O_2 (Fig. 1A). The death rates of hMSCs maintained in 10% FBS were not affected by 48-hour exposure to hypoxia (Fig.1A versus Fig. 2A). In addition, the increase in the cell death rates induced by decreasing the FBS concentration was similar with hMSCs exposed to either hypoxia or 21% O_2 (15 ± 7% and 13 ± 4% for 0% FBS, respectively).

Combined effects of FBS deprivation and scaffold substrate shift under short hypoxia. In order to simulate current procedures as closely as possible, the cumulative effects on the hMSC survival rates of serum deprivation and a shift to scaffold in a hypoxic environment were then investigated by repeating the above experiment (Combined effects of FBS deprivation under short hypoxia) after transferring hMSCs onto alumina (Fig. 2B) or calcium carbonate (Fig. 2C) substrates and exposing them to 48-hour hypoxia in αMEM containing either 0%, 1% or 10% FBS. Results were compared to those obtained when hMSCs were transferred from plastic to alumina or calcium carbonate substrates and exposed at 21% O_2 to αMEM containing either 0%, 1% or 10% FBS (Fig. 1B and 1C, respectively). Death rates of hMSCs seeded onto alumina or calcium carbonate substrates were affected neither by 48-hour exposure to hypoxia (Fig 1B versus 2B and Fig. 1C versus 2C) nor by decreasing the FBS concentration (Fig.2B and 2C).

In conclusion, the hMSC death rate was increased by serum deprivation (0% FBS) when hMSCs were transferred from plastic to plastic or alumina substrates but not when hMSCs were transferred from plastic to calcium carbonate substrate at 21% O_2. In addition, 48-hour exposure of hMSCs to hypoxia alone or combined with a shift to a scaffold substrate did not significantly affect the hMSC death rates as long as the serum concentration was maintained at 10%. Death rates were maximum under a complete serum deprivation.

Effects of FBS deprivation under prolonged hypoxic conditions.

Revascularization of an ischemic area of any significant size will take a long period of time. For instance, in the rabbit ear chamber model, the average rate of vascularization has been estimated to be 0.09 to 0.25 mm/day (29), which suggests that complete revascularization of an engineered construct 2 mm thick will take approximately 4 days in this model. Therefore, to better mimic the long-term ischemia that is bound to occur after transplantating MSCs, we further investigated the long-term effects of serum and oxygen deprivation on the hMSC death rates, by transferring hMSCs (expanded on plastic at 21% O_2 in αMEM/10% FBS) onto a plastic substrate and exposing them to hypoxic conditions in αMEM containing either 1% or 10% FBS for up to 120 hours.

Long-term hypoxia combined with serum deprivation (i.e. 1% FBS) had considerable cumulative effects on the hMSC death rate, as these conditions resulted in the complete death of the hMSCs (99 ± 1% at 120h; $p<0.0001$ in comparison with results obtained after exposure to 48-hour hypoxia) (Fig 3). However, the effects of long-term hypoxia were reduced by half when the hMSCs were exposed to hypoxia in the presence of 10% FBS. Under these conditions, the hMSC death rates dropped from 99 ± 1 to 51 ± 31%, but can still be said to be of considerable biological significance.

Discussion

Conventional MSC transplantation procedures involve expanding the cells at 21% O_2 in cell culture medium containing 10% FBS in an incubator at 37°C. The cells are then used in the form of cell suspensions (for cell therapy) or seeded into a scaffold (for tissue engineering). In all the approaches used so far, in vivo transplantation occurs in an ischemic environment where the initial lack of vascularization leads to sudden oxygen and nutrient deprivation. The aim of the present study was to investigate the effects of these parameters (substrate scaffold shift, oxygen and nutrient deprivation) on hMSC survival.

Substrate scaffold shifts from plastic to either plastic or alumina substrates had no effect on hMSC survival rates. Substrate scaffold shift from plastic to calcium carbonate substrate, however, eliminated the increase in cell death induced by serum deprivation. This could be explained either by (i) modulation of hMSC sensitivity to serum deprivation by calcium carbonate substrate or (ii) increase of cell death rates by calcium carbonate substrate concealing increase by serum deprivation. Both hypothesis may be explained by different surface properties between scaffold substrates, as several studies have shown that surface composition and properties can affect cell behavior (for a review, see (30-32)), and demonstrate that the choice of the substrate material used for cell therapy purpose could affect hMSC survival after transplantation.

In the present study, the effects of sudden serum and oxygen deprivation were assessed both separately and combined in order to determine the contribution of each parameter and to investigate whether either of these parameters may modulate the effects of the other. First, total serum withdrawal (0% FBS) at 21% O_2 increased the hMSC death rates (13%), whereas serum reduction (1% FBS) did not. These results suggest that FBS contains factors required for cell survival (besides the amino acids that were added under the 0% FBS conditions). Some clues about these survival factors may be found in studies conducted on serum-free culture systems as supplementation of cell culture medium with growth factor cocktails (33) or platelet rich plasma (34) is able to maintain viable and proliferative hMSCs. Second, hypoxia alone had no effect on cell death during an exposure time of 48 hours. These findings are in agreement with previous findings that exposure of primary hMSCs to hypoxic conditions (2% O2) did not affect cell death (35) or that chronic (3 weeks) exposure of rats to hypoxia (50 kPa) did not affect the colony-forming unit fibroblast activity of bone marrow derived MSCs (36). Park et al. have reported, however, that hypoxia (2% O_2) reduced cell viability in a human osteoblast-like cell line (37). These discrepancies suggest that primary cells such as hMSCs are less sensitive than cell lines such as MG63 to hypoxia. Long-term *in vitro* culture of cell lines and their acclimatization to a hyperoxic environment in comparison with

primary cells (21% O_2 in the cell culture incubator versus a maximum of 13% in the body) may explain why the former are more sensitive to reduced oxygen tensions.

When hypoxia and serum deprivation were combined, the cell death rates increased, but to the same extent as that observed with serum deprivation alone. These results suggest that between oxygen and nutrient deprivation, nutrient deprivation is the stronger factor and, as long as oxygen deprivation does not last for more than 48 hours, constitutes the main limitation for MSC survival after transplantation.

Surprisingly, when hypoxia was prolonged and combined with FBS deprivation (1% FBS), the hMSC death rates increased drastically, reaching 99% within 72 hours of exposure. These results are consistent with those observed by Toma et al., who reported that only 0.5% of hMSCs transplanted into an ischemic murine heart survived (7). Most interestingly, adding 10% FBS limited hMSC death rates (3% after 72 hours and 55% after 120 hours), which confirms that adding serum reduces the cell death induced by oxygen deprivation. Similar conclusions were reached by Zhu et al. after brief (24 hours) exposure of primary rat MSCs to nutrient (0% FBS) and oxygen (3% O_2) deprivation (38).

Although, the exact mechanisms underlying hMSC death as the results of oxygen or serum deprivation still remain to be elucidated, brief (24 hours) nutrient and serum starvation have been found to induce caspase dependent apoptosis (nuclear shrinkage, chromatin condensation, decrease in cell size, and loss of membrane integrity) (38). The relevance of these findings to human cells exposed to long-term serum and oxygen deprivation still remains to be determined.

In conclusion, the present findings show that hMSC survival is affected by sudden oxygen and nutrient deprivation but not by a scaffold shift. Exposure to hypoxia and serum starvation lasting for up to 72 hours (3 days) can lead to massive cell death. Serum deprivation seems, however, to be the stronger of the two factors as it increases the hMSC death rates when occurring separately, whereas this is not the case with hypoxia. These findings also suggest that the massive cell death observed after cell transplantation can be attenuated by adding survival factors to engineered constructs or cell suspensions at the time of transplantation. This could be done using fibrin gels containing either serum (FBS or autologous serum) or survival factors, as this natural polymer has been efficiently used as a cell scaffold for MSCs (39) and as a delivery system for delivering angiogenic growth factors (40, 41).

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Disclosure Statement

The authors declare no competing financial interests.

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Figure 1. Effects of FBS deprivation and scaffold substrate shift at 21% O2 on hMSC death rates.

hMSCs (expanded on plastic at 21% O_2 in αMEM/10% FBS) were seeded onto plastic (**A**), alumina (**B**) and calcium carbonate (**C**) substrates and left to adhere overnight. hMSCs were then exposed to medium containing either 0%, 1% or 10% FBS at 21% O_2 for 48 hours. 0% FBS medium was supplemented with amino acids 1% (0%+AA). Cell death rates were then assessed performing Live/Dead staining followed by image analysis. Values are means ± SD; n=5 donors; cells from each donor were tested in triplicate.

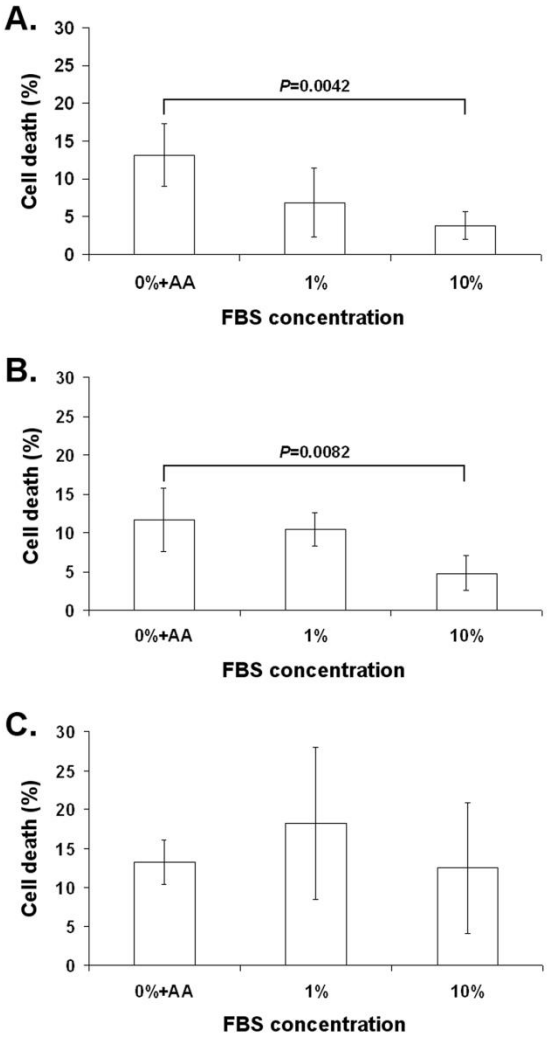

Figure 2. Effects of FBS deprivation and scaffold substrate shift under hypoxic conditions on hMSC death rates.

hMSCs (expanded on plastic at 21% O_2 in αMEM/10% FBS) were seeded onto plastic (**A**), alumina (**B**) and calcium carbonate (**C**) substrates and left to adhere overnight. hMSCs were then exposed to medium containing either 0%, 1% or 10% FBS under hypoxic (<1%O_2) conditions for 48 hours. 0% FBS medium was supplemented with amino acids 1% (0%+AA). Cell death rates were then assessed performing Live/Dead staining followed by image analysis. Values are means ± SD; n=5 donors; cells from each donor were tested in triplicate.

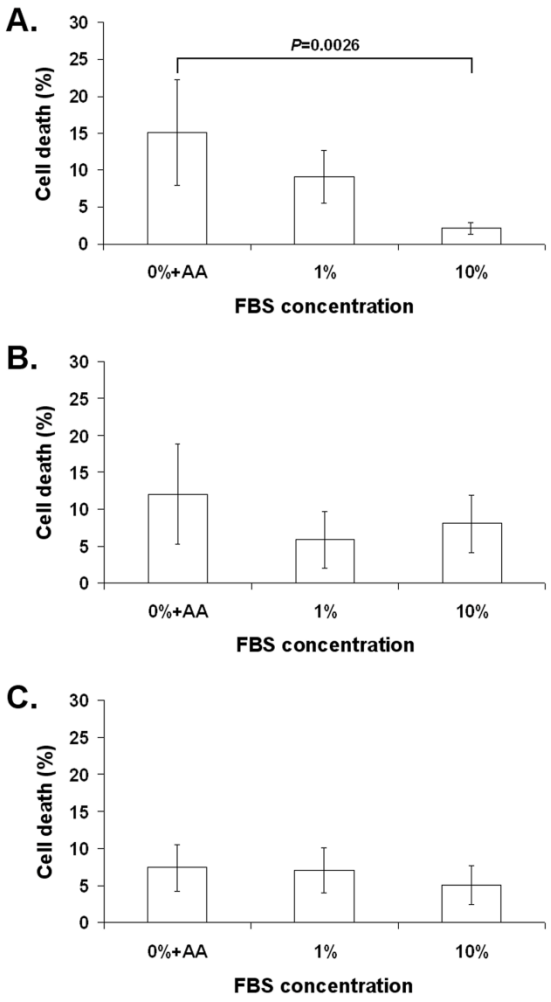

Figure 3. Effects of FBS deprivation under prolonged hypoxia on hMSC death rates.

hMSCs (expanded on plastic at 21% O_2 in αMEM/10% FBS) were seeded onto cell culture plastic substrate and left to adhere overnight. hMSCs were then exposed to medium containing either 1% (white bars) or 10% (grey bars) FBS under hypoxic (<1% O_2) conditions for 48, 72, and 120 hours. Cell death rates were assessed performing Live/Dead staining followed by image analysis. Values are means ± SD; n=5 donors; cells from each donor were tested in triplicate.